b. allowing the targeting moiety to circulate through the injected human or animal for a time sufficient to bind to the target analyte of interest and form a targeting moiety:target analyte conjugate which decreases the clearing rate of the target analyte;

c. obtaining a sample of blood from the human or animal after a defined period of time;

d. combining the sample of blood with a capture moiety [capable of binding] that binds specifically to the targeting moiety:target analyte conjugate in order to form an assay mixture;

e. incubating the assay mixture of step d to allow the capture moiety to bind [specifically] to the targeting moiety:target analyte conjugate and form targeting moiety:target analyte;capture moiety complexes in the assay mixture;

f. removing any unbound and unconjugated targeting moiety and target analyte from the assay mixture;

g. detecting the amount of bound labeled [targeting moiety:target analyte conjugate on the capture moiety];

h. wherein the amount of labeled [targeting moiety:target analyte conjugate bound to the capture moiety and detected] in step (g) provides a measure of the production of secreted target analyte in the sample during the defined period of time [determining the amount of the target analyte in the sample correlating to the amount of targeting moiety:target analyte conjugate bound to the capture moiety and detected in step (g); and

i. wherein the target analyte is a peptide or protein hormone.

4. (Once Amended) The method of claim 14 [3], wherein the target analyte [protein] is a cytokine.

6. (Twice Amended) The method of claim 4, wherein the cytokine is selected from the group consisting of interleukin-1, interleukin-2, interleukin-3, interleukin-4, interleukin-5, interleukin-6, interleukin-7, interleukin-8, interleukin-9, interleukin-10, interleukin-11, interleukin-12, interleukin-13, interleukin-14, interleukin-15,

interleukin-16, interleukin-17, interleukin-18,, interferon-alpha, interferon-beta, interferon-gamma, lymphotoxin, tumor necrosis factor-alpha, transforming growth factor (TGF)-beta, granulocyte macrophage-colony stimulating factor (GM-CSF), nerve growth factor (NGF), and epidermal growth factor (EGF).

7. (Twice Amended) The method of claim 1, wherein the blood is selected from the group consisting of whole blood, serum and plasma.

13. (Thrice Amended) The method of claim 1, wherein the targeting moiety is labeled with a small molecule hapten and wherein the method further comprises the step of binding the small molecule hapten [by linking the targeting moiety to a label which label can then be bound] to a binding partner which is conjugated to an enzyme.

14. The method of claim [13, wherein the label is a small molecule hapten] 1, wherein the set period of time is from about 1 hour to about 72 hours.

15. The method of claim 13 [14], wherein the hapten is biotin.

16. The method of claim 13, wherein the enzyme-conjugated binding partner is selected from the group consisting of streptavidin, anti- biotin antibody, anti-hapten antibody, and anti-immunoglobulin antibody.

17. The method of claim 13, wherein the enzyme is selected from the group consisting of alkaline phosphatase, glucose oxidase, beta -galactosidase, malate dehydrogenase, staphylococcal nuclease, delta-5-steroid isomerase, yeast alcohol dehydrogenase, alpha-glycerophosphate dehydrogenase, triose phosphate isomerase, horseradish peroxidase, asparaginase, ribonuclease, urease, catalase, glucose-6-phosphate dehydrogenase, glucoamylase and acetylcholinesterase.

20. (Twice Amended) The method of claim 8, further comprising after step (a) the step of injecting a second targeting moiety [wherein the targeting moiety is a first targeting moiety itself capable of being bound by a second targeting moiety] wherein the second targeting moiety is specifically bound [recognized] by the capture moiety.

25. (Once Amended) The method of claim 20, wherein the means for detecting the targeting moiety:target analyte;capture moiety complexes [bound conjugate on the solid support] is by radioimmunoassay[, wherein the molecule capable of binding the targeting moiety is labeled by linking the targeting moiety to a radioisotope].

26. (Twice Amended) The method of claim 20, wherein the second targeting moiety is detectably labeled by an enzymatic label [linking it to a label which label can then be bound to a binding partner which is conjugated to an enzyme].

31. (Once Amended) The method of claim 20, wherein the [molecule capable of binding the targeting] capture moiety is labeled by linking to a fluorescent labeling compound.

34. (Once Amended) A reagent kit useful in performing the method of claim 1, comprising

(a) a first reagent containing a labeled targeting moiety specific for the target analyte [and capable of forming a conjugate with the target analyte];

(b) a second reagent separated from said first reagent which contains a capture moiety specific for the target analyte even when conjugated with the targeting moiety [for said conjugate] and

(c) a third reagent separated from said first and second reagents which contains a standard for the analyte.

37. (Twice Amended) A reagent kit useful in performing the method of claim 20 [1], comprising: (a) a first container having first targeting moieties that immunoreact with a target analyte, and are operatively linked to a label; (b) a second container having second targeting moieties that immunoreact with the target analyte at a site different from the first targeting moieties but are not in the first container; and (c) one or more other containers comprising one or more of the following: a sample reservoir, a solid phase support, wash reagents, reagents for [capable of] detecting the presence of the first targeting moieties [bound antibody] from the second container, or reagents for [capable of] amplifying the label.

It is believed that it would be helpful to first review the main points of the present invention so that the critical elements can be understood and so that the claims may be amended to fully encompass the invention's contribution to the arts.

First, the present invention is an immunoassay for use in detecting and monitoring analyte production *in vivo*. Previously described methods use a binding molecule to bind the analyte and immediately measure the quantity of analyte present at a given time. The present methods provides for the injection of a targeting moiety to bind the analyte as it is produced so that after a set period of time, typically from about 1 hour to about 72 hours, a blood sample is taken from the subject for testing the amount of analyte secreted over the period of time. (see page 15, lines 3-10) The present invention provides the capability of measuring basal as well as stimulated hormone production.

Second, the present method preserves the analyte over the set period of time by preventing analyte destruction, utilization, and excretion so that analyte will accumulate in extracellular fluids, including blood. Because the labeled neutralizing targeting molecule binds the analyte causes the analyte to be slow clearing, this causes the analyte, which may normally have a very short in vivo half life, to accumulate in vivo as a targeting moiety:analyte conjugate. Therefore, in the present invention, cytokine-type secreted peptide or protein hormones can be accurately measured in the blood even in the presence of binding proteins that mask the hormone's activity in conventional assays. (see page 12, lines 15-19)

Specifically, the present invention provides a method of measuring the ***production*** of a target analyte (a secreted peptide or protein hormone) of interest in a human or animal.

It should be particularly noted that following injection and distribution of the labeled neutralizing targeting molecule, secreted target analyte will be bound by this molecule as the analyte is produced in vivo over time and will form a soluble complex, which will accumulate in blood and in other sites to which the labeled neutralizing targeting molecule has distributed. (see page 16, lines 24-29 and page 17, lines 1-7)

Prior methods have been unable to accurately measure analyte production, e.g., cytokines, because of rapid excretion, catabolism, and utilization of cytokines as well as the binding of cytokines to endogenous cytokine binding proteins, which can interfere

with detection. The present invention obviates all of these difficulties. (see page 5, lines 1-7) The main advantages are that this technique allows:

(1) The accumulation in serum of a secreted or shed biological material that normally has a short in vivo half-life. The use of a labeled antibody or other molecule that binds the biological material greatly facilitates the measurement of the biological material in serum. No other technique has been described that allows quantitation of the amount of a biological material that has been secreted over a fixed, definable period of time *in vivo*.

(2) Repeated measurements of analyte production over time to be made in individual humans or experimental animals; and

(3) Measurement of analyte production that is influenced little, if any, by the presence of endogenously produced soluble analyte receptors.

Previous methods were ineffectual because there is typically too little analyte present to measure in an instantaneous fashion. The present method takes a new approach to allow sufficient time for a sufficient amount of secretion of the analyte after injection to obviate this problem. In addition, it was previously believed in the art that one might not be able to get a targeting moiety to the required analyte or that to do so would require too much targeting moiety to be practical or that sufficiently large quantities would block the very processes being studied. Many peptide hormones have serum binding proteins or receptors that would again require impractical amounts of targeting moiety but it was surprisingly discovered that the present methods work as found.

We look forward to speaking with you later today to resolve the remaining issues with this application.

Respectfully submitted,

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